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Research Article

Detection of mutator phenotype in Brazilian patients with acute and chronic myeloid leukemia

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Abstract

The multisteps of tumorigenesis involve the classic chromosomal instability and the mutator phenotype pathways featured by a predisposition to acquire mutations in tumor suppressor genes and oncogenes. Expansion and contraction of microsatellite sequences due to a deficient mismatch repair system are a marker of the mutator phenotype. Controversial results regarding the extent of microsatellite instability (MSI) have been reported in the development and progression of myeloid malignancies. Here, we investigated MSI and loss of heterozygosity (LOH) frequencies at the microsatellite *loci BAT-26*, D7S486, D8S135, *ANK1*, *IFNA*, *TP53* and *bcr* of 19 Brazilian patients with acute (AML) and chronic myeloid leukemia (CML). One AML patient and one CML patient were categorized as having a high degree of microsatellite instability (MSI-H), corresponding to 10.5% (2/19) of all patients. LOH at *loci BAT-26* and *TP53* was present in 30% of the patients with AML alone. Despite the small sample size, our results suggest that the mutator phenotype, as verified by MSI frequency, could play a role in the leukemogenesis of a small subset of patients with myeloid leukemia.

Key words: loss of heterozygosity, microsatellite instability, mismatch repair, mutator phenotype, myeloid leukemia.

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Introduction

Leukemia is a proliferative disorder of the leukopoietic cells. The complex origin and nature of this disorder allow its classification into acute and chronic myeloid or lymphoid types (Harris *et al*., 2000). The multistep pathways of tumorigenesis involve the classic chromosomal instability pathway featured by chromosomal loss and LOH of tumor suppressor genes. In another possible pathway, a multistep mutator phenotype leads to the accumulation of mutations in tumor suppressor genes and oncogenes, due to a deficiency in the DNA mismatch repair system [MMR] (Peltomaki, 2001). Patients with such a predisposition to acquire mutations are likely to have cancer early in life, more than one primary tumor, and a family history of cancer (Ben-Yehuda *et al*., 1996).

Cells harboring a proficient MMR system can correct nucleotide mismatches during DNA replication or recombination and trigger apoptosis following serious DNA damage (Aquilina and Bignami, 2001). Deficiency to repair nucleotide mismatches causing loops in the template DNA strand during replication is associated to deletions of nucleotides in microsatellite *loci*. On the other hand, deficiency to repair the new DNA strand is related to the length expansion of microsatellite *loci* (Naidoo and Chetty, 1999). Microsatellites are highly polymorphic markers composed by short tracts of nucleotide repeats, dispersed throughout the human genome and prone to mutations by replication slippage. Increased frequency of somatic variation in the length of these markers, known as microsatellite instability

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(MSI), is a clear evidence of mutator phenotype (Aquilina and Bignami, 2001).

Controversial results regarding the extent of MSI have been reported in the development and progression of myeloid malignancies. This disagreement may be due to the number of patients studied, panel of markers chosen, MSI assessment criteria, source of non-tumoral control sample or methodological differences (Wada *et al*., 1994; Pabst *et al*., 1996; Mori *et al*., 1997; Tasaka *et al*., 1997; Boyer *et al*., 1998; Auner *et al*., 1999; Rimsza *et al*., 2000; Das-Gupta *et al*., 2001; Ribeiro *et al*., 2002). In this context, considering the scarcity of reports regarding such frequencies in Brazil, we aimed to investigate microsatellite alterations and the role of the mutator phenotype in a group of Brazilian patients with myeloid leukemia.

Materials and Methods

Patients and samples

Peripheral blood samples from 10 patients with acute and nine patients with chronic myeloid leukemia were collected in heparin at the Hematology Service of *Hospital das Clínicas* (Federal University of Goiás, Brazil). Additionally, exfoliated cells from the oral mucosa of every patient were obtained by gently scraping the inside lining of the mouth with a spatula. Patients were not selected based on stage, type nor therapy of the disease. Samples were collected from the patients following informed consent, according to the guidelines approved by the National Health Council of the Brazilian Ministry of Health.

DNA isolation

DNA samples were obtained from peripheral blood cells of all 19 patients and from bone marrow cells of two patients. DNA was isolated using conventional phenol-chloroform extraction and ethanol precipitation methods (Sambrook *et al*., 1989). Control DNA was extracted from the exfoliated buccal cells, using RapidPrep Micro Genomic DNA isolation Kit for Cells and Tissues (Amersham Biotech Inc., Piscataway, NJ) according to the manufacturer's instructions.

Microsatellite analysis

DNA amplification by polymerase chain reaction (PCR) was performed in a final volume of 25 µL containing 100 ng of genomic DNA, 50 mmol/L KCl, 10 mmol/L Tris HCl (pH 8.3), 1.5 mmol/L MgCl, 0.2 mmol/L each dNTP, 0.4 µmol/L of each sense and antisense primers for individual *locus*, and 1 U of Taq Polymerase (Amersham Biotech Inc.). A panel of seven microsatellite markers was used in this study (see Table 1). The touchdown thermal protocol included denaturation at 92 °C for 1 min, annealing at 58-48 °C (decreasing 1 °C/cycle for 10 cycles, followed by 15 cycles at 48 °C) for 1 min, and extension at 72 °C for 1 min. PCR products were visualized using an 8% polyacrylamide gel stained with 0.5 µg/mL ethidium bromide. Gels were analyzed using a VDS system (Amersham Biosciences Inc., Umeå, Sweden) and the TotalLab software version 1.0 (Nonlinear Dynamics Ltd., Newcastle Upon Tyne, UK) to determine band sizes (bp) and densities. Decrease in the density of any given heterozygous allele was measured as arbitrary units of pixel concentration. The fractional allelic loss (FAL) was calculated using the formula FAL = CL/CI, where CL is the number of chromosome arms lost and CI is the overall number of informative chromosome arms.

Statistical analysis

Means of MSI or LOH were analyzed using Fisher's exact test with an overall significance level of 0.05 (95% CI) for comparisons between the two means.

Table 1 - Microsatellite markers.

Marker	Location	Repeat motif	Primer sequence (5' - 3')	Reference
BAT-26	2p16	$(A)_n$	TGA CTA CTT TTG ACT TCA GCC AAC CAT TCA ACA TTT TTA ACC C	Parsons *et al*., 1995
D7S486	7q31.1	$(CA)_n$	AAA GGC CAA TGG TAT ATC CC GCC CAG GTG ATT GAT TGA TAG TGC	Goossens *et al*., 2003
D8S135	8p21.3-11	$(CA)_n$	GGG AGG CTT TAT AAT TAT TTA GC CTG GGC AAC AGA GTG GGA C	Wood *et al*., 1991
ANK1	8p11-21.1	$(CA)_n$	TCC CAG ATC GCT CTA CAT GA CAC AGC TTC AGA AGT CAC AG	Özcan *et al*., 2003
IFNA	9q22	$(CA)_n$	TGC GCG TTA AGT TAA TTG GTT GTA AGG TGG AAA CCC CCA CT	Kwiatkowski and Diaz, 1992
TP53	17p13.1	$(CA)_n$	ATT CCT TCT GGG CCC TTT C TGC CTA CTG CTC CAA CTT CA	Jones and Nakamura, 1992[1]
bcr	22q	$(CA)_n$	TGT TTT AAA TTT CAG AGC CTG G CGG CCT ATG TAT ATT TTC ACA CAT	Chissoe *et al*., 1995[1]

[1]Reference of the DNA sequence used to design the primer sets.

Results

MSI and LOH were investigated for seven microsatellite markers scattered over six different chromosomes of AML and CML patients. MSI was identified as a variation in the length of microsatellite alleles in the leukemic sample, as compared to the corresponding constitutional material from buccal cells. Seven out of 109 paired PCR amplifications exhibited instability at the *loci BAT-26*, D7S486, D8S135, *ANK1* and *TP53* (Figure 1). Instabilities were verified in two patients with AML and two with CML, corresponding to approximately 21% (4/19) of patients with MSI . Two of these four patients had a high degree of MSI (MSI-H ≥ 30% of assessed *loci*), suggesting the occurrence of the mutator phenotype. The other two patients with MSI had a low degree of MSI (MSI-L ≤ 30% of assessed *loci*), suggesting the occurrence of another pathway of leukemogenesis rather than the mutator phenotype. Table 2 shows details of the results for each patient.

The criterion for LOH identification was the absence or a decrease to less than 50% in the intensity of one heterozygous band. The authors were aware of LOH misclassification due to technical artifacts, such as preferential amplification or amplification failure due to DNA degradation and low amount of template. However, DNA preparations of good quality were available, and only heterozygous markers were considered informative. By using this criterion, markers *bcr* and D7S486 were heterozygous in most patients and, consequently, highly informative for LOH. Amplification of 31 paired sets of informative alleles from AML patients permitted the detection of two LOH events for marker *TP53* and another two for marker *BAT-26* (Figure 1). LOH events were found in three patients with AML, corresponding to 30% (3/10) of the AML patients. However, no LOH was detected in the 29 paired sets of informative alleles from CML patients (Table 2). Background data and statistic details for MSI and LOH are presented in Table 3.

Discussion

We studied 19 patients with myeloid leukemia and found 10.5% (2/19) of them with MSI–L and 10.5% (2/19) with MSI-H, which suggested the occurrence of the mutator phenotype in two of our patients (A11 and C07). It is noteworthy that MSI and LOH were not associated in any patient, corroborating the hypothesis that these two pathways of tumorigenesis do not occur together (Ponz de Leon *et al*., 1999). Additionally, it has been suggested that MSI is an adverse prognostic factor for leukemia and lymphoma patients, associated with short-term relapse and resistance to chemotherapy (Indraccolo *et al*., 1999). The association

Table 2 - Analysis of MSI and LOH in AML and CML patients.

Patient code	Type of leukemia	Sex/Age	FAL	Microsatellite *loci*						
				BAT-26	D7S486	D8S135	*ANK1*	*IFNA*	*TP53*	*bcr*
A05	AML	F/68	0.000	NI	#	NI	NI	-	-	#
A11	AML	F/39	0.000	NI	-	MSI	NI	#	MSI	-
A15	AML	F/50	0.000	#	-	NI	NI	#	NI	-
A17	AML	F/35	0.000	#	-	NI	-	-	-	-
A20	AML	F/18	0.000	NI	MSI	NI	NI	#	NI	-
A22	AML	F/22	0.000	NI	-	NI	#	#	-	-
A23	AML	F/41	0.125	NI	-	NI	-	#	LOH	-
A24	AML	F/43	0.167	NI	-	NI	NI	NI	LOH	-
A25	AML	F/61	0.000	NI	-	NI	-	-	NI	-
A28	AML	F/60	0.250	LOH[1]	-	NI	-	NI	-	-
C03	CML	F/45	0.000	NI	-	NI	NI	-	-	-
C05	CML	M/76	0.000	NI	-	NI	NI	NI	NI	-
C06	CML	M/34	0.000	#	-	NI	-	-	-	-
C07	CML	M/34	0.000	NI	-	MSI	MSI	#	MSI	-
C16	CML	M/49	0.000	-	#	NI	#	#	-	#
C17	CML	F/41	0.000	MSI	-	NI	-	-	-	-
C18	CML	M/43	0.000	#	-	NI	#	#	-	#
C19	CML	F/68	0.000	#	-	NI	-	NI	-	-
C20	CML	F/49	0.000	#	-	#	#	#	NI	-

AML = acute myeloid leukemia; CML = chronic myeloid leukemia; LOH = loss of heterozygosity; MSI = microsatellite instability; NI = not informative; # = not performed reaction; - = absence of MSI or LOH; [1] = double allele deletion.

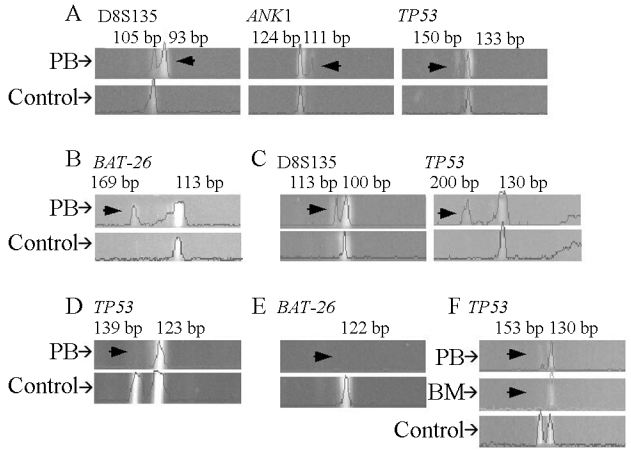

Figure 1 - Demonstration of MSI and LOH in AML and CML patients. MSI events are represented for CML patients C07 (A) and C17 (B) and for AML patient A11 (C). LOH events are represented for AML patients A24 (D), A28 (E), and A23 (F). MSI and LOH were assessed by comparison of the microsatellite alleles amplified from peripheral blood (PB) and bone marrow (BM) with alleles from buccal cells (control). Arrows indicate gain of new alleles (MSI) or allele loss (LOH) as compared to control DNA. MSI of patient A20 for *locus* D7S486 is not shown.

of mutator phenotype and *TP53* molecular inactivation, as reported by Ben-Yehuda *et al*. (1996) and Zhu *et al*. (1999), might be an alternative pathway for *de novo* leukemogenesis in elderly people or for an accentuated level of genomic instability in therapy-related leukemia patients. Both hypotheses are thought to indicate a rather negative prognosis. Moreover, an increased incidence of adverse cytogenetic abnormalities has been found in AML patients with MSI, and even a patient with favorable cytogenetic findings, but with MSI, was reported to have had early relapse of AML (Das-Gupta *et al*., 2001). In contrast, in CML patients, MSI has been identified as an uncommon event (Mori *et al*., 1997; Auner *et al*., 1999; Ribeiro *et al*., 2002), although there is one former report suggesting that MSI may occur as a late event in the evolution of CML toward blast crisis (Wada *et al.*, 1994).

In conformity with these previous findings, which correlate MSI and mutator phenotype with a more aggressive progression of AML, the prognosis of our AML patient (A11) with MSI-H was poor. The patient was initially diagnosed with myelodysplastic syndrome, which progressed to AML. Remission was successfully induced by chemotherapy, but the patient relapsed 17 months later and died during the second remission induction. On the other hand, our findings of MSI-H in CML were associated with a good prognosis. The CML patient C07 was treated with busulfan for six years, when the disease converted to the accelerated phase. The busulfan treatment was then replaced by hydroxyurea, and the disease has been under control for the last eight years. Cytogenetically this patient was found to be positive for the Ph chromosome, and bone marrow transplantation could not be done due to the lack of a histocompatible donor.

In the present study, the mean age of AML patients was 44 years (ranging from 18 to 68 years), and of CML patients 49 years (range: 34 to 76 years). Those of our patients who had MSI-H were 34 and 39 years old, while those who had MSI-L were 18 and 41 years old. It is of interest that our AML patients with MSI were younger than those studied

Table 3 - MSI and LOH frequencies in AML and CML reported elsewhere.

Type of leukemia (Nr. of cases)	Nr. of *loci*	MSI and LOH frequencies	p value[1]	Reference
AML (14)	5	7.1% of the patients had MSI	0.31	Ribeiro *et al.*, (2002)
CML (29)		0% of MSI for all patients	0.05	
AML (52)	11	10% of the patients had MSI	0.32	Das-Gupta *et al.*, (2001)
AML (132)	3	0% of MSI for all patients	< 0.01	Rimsza *et al.*, (2000)
CML (48)	12	0% of MSI for all patients	0.01	Auner *et al.*, (1999)
AML (39)	14	0% of MSI for all patients	0.04	Boyer *et al*., (1998)
		0% of LOH at the *locus BAT-26*	0.03	
CML (30)	82	0% of MSI for all patients	0.04	Mori *et al.,* (1997)
		3.3% of LOH at the *locus TP53*	0.78	
		0% of LOH at the *locus D7S486*	1.00	
AML (17)	69	5.9% of MSI at the *locus TP53*	0.48	Tasaka *et al.*, (1997)
		35% of the patients had MSI in $(CA)_n$ motifs	0.33	
AML (36)	22	5.6% of the patients had MSI in $(CA)_n$ motifs	0.16	Pabst *et al.*, (1996)
		5.5% of LOH at 2p	0.20	
CML (21)		14% of the patients had MSI in $(CA)_n$ motifs	0.31	
CML (39)	5	35.9% of the patients had MSI	0.35	Wada *et al.*, (1994)

[1]p value for Fisher's exact test with an overall significance level of 0.05 (95% CI) for the two means comparison.

by Das-Gupta *et al*. (2001), who reported that MSI in AML was restricted to elderly patients. It was suggested that exposure to environmental mutagens over a long period of time could lead to an increased frequency of MSI in elderly AML patients, due to defective MMR (Zhu *et al*., 1999; Das-Gupta *et al*., 2001). However, our findings of MSI-H were not associated with any history of chronical exposure to environmental mutagens.

All three new alleles found in the AML patients had greater lengths than those of the respective control sample, while CML patients had two larger and two smaller new alleles in the tumoral tissue, as compared to their corresponding wild-type controls (Figure 1). These new alleles with an increased length are an evidence of enhanced MMR deficiency in the new strand, immediately post DNA replication of leukemia cells (Naidoo and Chetty, 1999). This expansion of repetitive sequences may be part of a potential third tumorigenesis pathway characterized by epigenetic regulation. The epigenetic mechanisms regarding hematopoietic malignancies have been described as being likely to inactivate tumor suppressor genes by methylation of their promoter regions (Chim *et al*., 2002).

The absence of amplification of the *BAT-26 locus* of patient A28 suggested that a double allele deletion had occurred. Also, polymorphisms or mutations in the primer-binding site of one or both alleles could result in allele amplification failure or even the finding of null alleles. The possibility of amplification failure due to sample artifact was excluded after experimental repetitions and successful amplification of other six *loci* using the same DNA sample. Although the *BAT-26 locus* is an intronic site of the MMR gene *hMSH2* (Rimsza *et al*., 2000), the lack of MSI in that patient may be due to some enzymatic overlap to repair DNA mismatches (Aquilina and Bignami, 2001). LOH of *TP53* was found in our study with FAL values of 0.125 and 0.167 for patients A23 and A24, respectively. Once leukemia patients are highly expected to have chromosomal aberrations (Willman, 1999), the frequency of LOH in our study could be an underestimate, because only seven *loci* were surveyed to screen six different chromosomes. Additionally, contaminating normal cells could have obscured LOH assessment (Mori *et al*., 1997).

The MSI frequencies found in the present study were statistically different ($p < 0.05$, Fisher's exact test) from those found by Mori *et al*. (1997), Boyer *et al*. (1998), Auner *et al*. (1999) and Rimsza *et al*. (2000), who reported absence of MSI in myeloid leukemias. However, there was no statistical difference from some frequencies reported earlier by Wada *et al*. (1994), Pabst *et al*. (1996), Tasaka *et al*. (1997), Das-Gupta *et al*. (2001) and Ribeiro *et al*. (2002), whose findings demonstrated MSI in patients with myeloid leukemia. A statistical difference was also observed between the LOH frequency reported by Boyer *et al*. (1998) for the marker *BAT-26* in AML patients and the frequency found in this study. However, there was no statistical difference between our LOH frequencies and those reported for *loci* D7S486 and *TP53* (Mori *et al*., 1997) and also for some regions of the short arm of chromosome 2 described by Pabst *et al*. (1996).

In this and in most of the studies focusing on hematological malignancies, MSI was not a general phenomenon, supporting that MSI is not suitable for differential diagnosis among different types of leukemia. Moreover, because tumor samples at diagnosis were not available, the roles of MSI and the mutator phenotype in the onset of myeloid leukemia in our patients were not clarified. Nonetheless, our findings regarding the mutator phenotype in myeloid leukemia suggest that this phenotype could play a role, if not in the onset and development, at least in the progression stage of leukemogenesis in a small subset of myeloid leukemia patients.

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